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Restoration of adipose function in obese, glucose-tolerant men following pioglitazone treatment is associated with CCAAT enhancer-binding protein β upregulation.

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Adipose Functionality and Pioglitazone

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Abstract

Obese adipose tissue (AT) exhibits increased macrophage number. Pro-inflammatory CD16⁺ peripheral monocyte numbers are also reported to increase with obesity. The current study was undertaken to simultaneously investigate obesity-associated changes in CD16⁺ monocytes and adipose tissue macrophages (ATM). In addition, a pilot randomised placebo controlled trial using the peroxisome proliferator-activated receptor (PPAR) agonists, pioglitazone and fenofibrate was performed to determine their effects on CD14⁺/CD16⁺ monocytes, ATM and cardiometabolic and adipose dysfunction indices. Obese glucose-tolerant men (n=32) were randomised to placebo, pioglitazone (30 mg/day) and fenofibrate (160 mg/day) for 12 weeks. A blood sample was taken to assess levels of serum inflammatory markers and circulating CD14⁺/CD16⁺ monocyte levels via flow cytometry. A subcutaneous (sc) AT biopsy was performed to determine adipocyte cell surface and AT macrophage (ATM) number, the latter was determined via assessment of CD68 expression by immunohistochemistry (IHC) and real time PCR. SC AT mRNA expression of CCAAT enhancer-binding protein β (CEBP β), sterol regulatory element-binding protein 1c (SREBP1c), PPAR γ 2, insulin receptor substrate 1 (IRS-1), glucose transporter type 4 (GLUT 4) and tumour necrosis factor α (TNF- α) were also assessed. Comparisons were made between obese and lean controls (n=16) at baseline, and pre- and post-PPAR agonist treatment. Obese individuals had significantly increased adipocyte cell surface, % CD14⁺/CD16⁺ monocyte numbers and ATM number (all $p=0.0001$). Additionally, serum TNF- α levels were significantly elevated ($p=0.017$) and adiponectin levels reduced (total: $p=0.0001$; high: $p=0.022$) with obesity. ATM number and % of CD14⁺/CD16⁺ monocytes correlated significantly ($P=0.05$). Pioglitazone improved adiponectin levels significantly ($p=0.0001$), and resulted in the further significant enlargement of adipocytes ($p=0.05$), without effect on % CD14⁺/CD16⁺ or ATM number. Pioglitazone treatment also significantly increased sc AT expression of CEBP β mRNA. The finding that improvements in obesity-associated insulin resistance following pioglitazone were associated with increased adipocyte cell surface and systemic adiponectin levels, supports the centrality of AT to the cardiometabolic derangement underlying the development of T2D and CVD.

Introduction

It has been reported that in obesity adipose tissue (AT) macrophage number increase, in addition to undergoing a phenotypic switch from protective M2 to pro-inflammatory M1 [1-3]. Evidence is mounting to support the contention that paracrine interactions between AT macrophages (ATM) and adipocytes play a central role in initiating and maintaining obesity-associated adipose dysfunction [1-4]. It has been speculated that AT dysfunction contributes to the systemic inflammatory status of obesity and, thus, may promote the development of type 2 diabetes (T2D) and cardiovascular disease (CVD). Therefore, targeting the underlying inflammatory mechanisms may have therapeutic potential in the management of obesity and subsequent CVD risk.

Excess energy intake results in impaired adipogenesis, enlargement of adipocytes and increased secretion of pro-inflammatory adipokines, in addition to a decreased production of the insulin sensitising adipokine, adiponectin [1]. Circulating levels of adiponectin have been considered a surrogate marker of adipose functionality and in terms of specific risk biomarkers for CVD and diabetes, a recent publication examining the contribution of different biological pathways to the development of type 2 diabetes concluded that adiponectin was the most important contributor, explaining one third of the risk [5]. Pro-inflammatory adipokines, which include TNF- α , interleukin 6 (IL-6) and monocyte chemoattractant protein-1 (MCP-1), modulate insulin sensitivity, cardiovascular homeostasis, inflammation as well as adipose mass [6]. Large adipocytes release more saturated free fatty acids which can stimulate macrophages to increase TNF- α production via NF- κ B activation [7,8]. In turn macrophage-derived TNF- α activates the adipocytes inducing further lipolysis and secretion of chemokines such as MCP-1 which promote the diapedesis of peripheral monocytes into the AT and differentiation into macrophages. The pro-inflammatory CD16⁺ peripheral monocytes are thought to drive the inflammatory processes associated with atherosclerosis. A significant association between CD16⁺ monocytes and both obesity and subclinical atherosclerosis has been reported [9]. More recently it has been observed that weight loss can diminish this monocyte subpopulation [10].

Peroxisome proliferator-activated receptor (PPAR) ligands were developed to improve insulin sensitivity however they demonstrate additional effects on the arterial wall, which suggest that they also could reduce cardiovascular risk. Previously we have demonstrated that pioglitazone and fenofibrate treatment of obese, glucose tolerant men reduces inflammation, improves markers of endothelial function and reduces arterial stiffness [11]. Furthermore the improved insulin sensitivity observed with pioglitazone treatment was accompanied by increased adiponectin demonstrating the potential of PPAR agonists to reduce the incidence of premature CVD associated with obesity through effects on the arterial wall and AT. Recent reports indicate that pioglitazone has direct effects on subcutaneous (sc) AT promoting adipogenesis in obese, non-diabetic, insulin-resistant subjects [12, 13]. Fenofibrate has also been shown to reduce fat mass through increased β -oxidation in various animal models [14, 15], but information regarding the effects of fenofibrate in human sc AT is lacking.

While previous investigations have investigated obesity-associated changes in ATM number or CD16⁺ monocytes, to date these monocyte/macrophage populations have not been examined within a single study. Therefore, the current study was undertaken to simultaneously examine obesity-associated changes in peripheral CD 14⁺/CD16⁺ monocytes and ATM in order to assess potential relationships between these monocyte/macrophage populations, and between these and cardiometabolic/adipose dysfunction indices in a normoglycaemic, but insulin resistant, obese population. Moreover, in order to investigate the effects of PPAR agonists, pioglitazone and fenofibrate on CD 14⁺/CD16⁺ monocytes, ATM number, adipocyte cell surface and sc AT gene expression, a pilot randomised placebo controlled clinical trial was conducted.

Methods

Study design

The protocol for the randomised placebo controlled trial was approved by Office for Research Ethics Committees Northern Ireland (reference number 06/NIR03/146) and clinical trial details were logged in the EudraCT database (reference number 2006-004296-35). Clinical Trial Authorisation was obtained from the Medicines and Healthcare Products Regulatory Agency.

Setting and participants

Obese [body mass index (BMI) ≥ 30 kg/m²], glucose tolerant males, aged 35-65 years and lean controls were recruited from the general population (including General Practice patients, hospital and university staff) by clinical trial staff. All lean and obese participants attended the Regional Centre for Endocrinology and Diabetes at the Royal Victoria Hospital for assessment and gave written informed consent. Exclusion criteria were as follows: smoker, clinical cardiac disease, clinical dyslipidaemia, plasma cholesterol >7 mmol/L, fasting triglycerides (TGs) >5 mmol/L, blood pressure $>160/90$ mmHg, diabetes/family history of diabetes, glucose intolerance, or use of hypertensive, cardiac, non-steroidal anti-inflammatory drugs or lipid-lowering therapies.

Pre-treatment visit: All lean (n=16) and obese (n=37) participants attended hospital after an overnight fast for a medical history and examination, including weight, height and waist:hip ratio (WHR) determination. Blood pressure was measured at the right brachial artery using the OMRON HEM-705CP automated sphygmomanometer (OMRON, Milton Keynes, Bucks, UK). A 75 g oral glucose tolerance test (OGTT) was performed with plasma glucose samples taken at 0, 30, 60, 120 and 180 minutes to determine glucose tolerance and insulin sensitivity. The homeostatic model assessment of insulin resistance (HOMA) index was calculated using the following formula: fasting insulin (mU/L) x fasting glucose (mmol/L) / 22.5. Blood was drawn and aliquoted for lipid profile, haemoglobin A_{1c} (HbA_{1c}), monocyte isolation, serum adiponectin and TNF- α . A sc fat biopsy was also obtained.

Treatment phase: Obese subjects taking part in the pilot clinical trial (n=28) were randomised to 12 weeks treatment with either fenofibrate (Supralip®, Fournier) 160 mg once per day, pioglitazone hydrochloride (ACTOS®, Takeda) 30 mg once per day or placebo. For safety purposes, liver function tests were also performed every 4 weeks.

Post-treatment visit: Obese subjects randomised to 12 weeks treatment attended for a post-treatment assessment at which all pre-treatment assessment procedures were repeated.

Biochemical and lipid assessment

HbA_{1c} was assessed by ion-exchange high-performance liquid chromatography on an Adams™ HA-8160 automated analyser (Menarini Diagnostics, Wokingham, Berkshire, UK). Serum insulin was measured by immunoassay on an Abbott IMx analyser (Abbott Diagnostics, Maidenhead, Berkshire, UK). Fasting plasma glucose, total cholesterol (TC), HDL-cholesterol (HDL-C) and TGs were measured using slide based dry chemistry on a VITROS 950 analyser (Ortho-Clinical Diagnostics, Bucks, UK). LDL-cholesterol (LDL-C) was calculated using the Friedewald equation.

Analysis of serum inflammatory markers

Total and high adiponectin were analysed using a human multimeric adiponectin ELISA (ALPCO immunoassays, Newmarket, Suffolk, UK; intra-assay CV total 1.30%, high 2.04%; inter-assay CV total 3.50%, high 2.50%). TNF- α was measured using the Quantikine® high sensitivity human TNF- α immunoassay kit (R&D systems, Abingdon, UK; intra-assay CV 3.67%; inter-assay CV 6.40%).

Preparation and analysis of CD14⁺/CD16⁺ peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were isolated by Histopaque (density 1.077 g/L; ratio of blood to Histopaque, 1:1) (Sigma-Aldrich, Dorset, UK) and incubated for 30 min at 4°C with two fluorescently labelled monoclonal antibodies: FITC-conjugated anti-CD14 (Santa Cruz Biotechnology, Heidelberg, Germany) and PE-conjugated anti-CD16 (Santa Cruz Biotechnology). Cells were then washed in phosphate buffered saline (PBS), centrifuged for 10 min at 250 g and resuspended in PBS/4% paraformaldehyde (PFA) 4:1 (v/v). Fixed CD14⁺/CD16⁺ cells were analysed via flow cytometry (FACSCalibur) (Becton Dickinson, Oxford, UK). Monocytes were identified by

forward and side scatter properties. Fluorescence data were collected on 10,000 cells and analysed using CELLQUEST software (Becton Dickinson).

Adipose analysis

Subcutaneous fat biopsy: Skin was firstly cleaned and anesthetized with 2 % xylocaine. A blunt dissection was then made and a small sample of sc AT (approximately 1 g) was removed from the periumbilical region using sterile forceps. Collected tissue was washed extensively in PBS and divided into 2 aliquots: i) an aliquot was formalin fixed and paraffin embedded; ii) an aliquot was added to RNAlater (Ambion, Warrington, UK), snap frozen and stored at -80°C until required.

Adipocyte cell surface: Formalin fixed paraffin embedded sc AT sections ($4\text{ }\mu\text{m}$) were stained in haematoxylin and photographed in triplicate using an Olympus microscope and SPOT Advanced software (SPOT Imaging Solutions, Sterling Heights, Michigan, USA). Captured images were examined using Photoshop CS4 Extended software (Adobe Systems Incorporated, San Jose, USA). The magnetic lasso tool was used to outline adipocytes and from this the software calculated cell area in pixels. Pixels were converted manually to μm^2 in excel using a scale conversion of $1\text{ pixel}=2.3543\text{ }\mu\text{m}^2$ [11].

ATM assessment

ATM content was assessed by both immunohistochemistry (IHC) staining for CD68 in formalin fixed paraffin embedded sections, and real time polymerase chain reaction (PCR) analysis of CD68 messenger RNA (mRNA) expression, in recognition that this combinatorial approach largely overcomes the limitations of using either method in isolation [16]. Several studies have reported a strong correlation between results obtained via CD68 IHC (Mphi, fraction of CD68 expressing cells) and mRNA expression of CD68 when used to assess of AT macrophage number [16-18].

IHC analysis: Sections ($4\text{ }\mu\text{m}$) of formalin fixed paraffin embedded sc AT were mounted onto APES-coated slides, dewaxed in xylene and incubated with a heated citrate buffer (100°C , 20 min) for antigen retrieval. An avidin/biotin blocking reagent [(Avidin/Biotin Blocking Kit; SP-2001) Vector Laboratories, Peterborough, England] was then applied to the sections, according to the manufacturer's protocol, before being incubated with hydrogen peroxide for 5 min. Sections were covered with a CD68 PGM1 IgG3 Kappa primary antibody (N1576; Dako, Cambridgeshire, UK) and incubated for 20 min. Negative control sections were incubated in a universal negative control reagent (N1698; Dako). For staining and development of the sections, a DakoCytomation LSAB 2 System-HRP Kit (K0673; Dako) was used, exactly according to the manufacturer's instructions. The number of CD68 positive cells were expressed as a percentage of the total number of cells counted per slide per subject.

Real time PCR: Total RNA was extracted from frozen whole sc AT using the RNeasy® Lipid Tissue Midi Kit (Qiagen, Crawley, West Sussex, UK), exactly according to the manufacturers protocol. RNA quantity and purification was determined on a ND-1000 NanoDrop® spectrophotometer (Thermo Scientific, USA). Total RNA (100 ng) was reversed transcribed using the Transcriptor High Fidelity complementary DNA (cDNA) Synthesis Kit (Roche, Burgess Hill, West Sussex, UK), according to the manufacturer's instructions and samples were stored at -20°C until required.

Real-time PCR was performed using FastStart SYBR Green mastermix (Roche) on ABI Prism 7000 sequence detection system (Applied Biosystems, Paisley, UK) programmed for universal cycling conditions (95°C for 10 min and 40 cycles of 95°C for 15 s and 60°C for 1 min) followed by melting curve analysis. Gene specific PCR primers were designed using the Primer Express software version 1.5 (Applied Biosystems) and synthesized by Invitrogen Life Technologies. The primers used were as follows: CD68 (GenBank accession no. NM_001251; forward 5'-CCCCACGCAGCACAGTG-3'; reverse 5'-GATCTCGAAGGGATGCATTCTG-3'), CCAAT enhancer-binding protein β (CEBP β) (GenBank accession no. NM_005194; forward 5'-GCCGCCGCCTGCCTTTAAATC-3'; reverse 5'-GCCAAGCAGTCCGCCTCGTAG-3'), PPAR γ 2 (GenBank accession no. NM_015869; forward 5'-GGCCAAGGCTTCATGACAAG-3'; reverse 5'-AAAAGGCTTTCGCAGGCTCT-3'), sterol regulatory element-binding protein 1c (SREBP1c) (GenBank accession no. NM_001005291; forward

5'-TGCAACACAGCAACCAGAAAC-3'; reverse 5'-TTGCTTTTGTGGACAGCAGTG-3'), glucose transporter type 4 (GLUT 4) (GenBank accession no. NM_001042; forward 5'-ATGTTGCGGAGGCTATGGG-3'; reverse 5'-GGAGGACCGCAAATAGAAGGA-3'), insulin receptor substrate 1 (IRS-1) (GenBank accession no. NM_005544; forward 5'-TGAGGATTTAAGCGCCTATGC-3'; reverse 5'-TTGAGCTACTGACGGTCCTCTG-3') and TNF- α (GenBank accession no. NM_000594; forward 5'-ATCTTCTCGAACCCCGAGTGA-3'; reverse 5'-GGGTTTGCTACAACATGGGC-3'). PCR was performed in triplicate with cycle threshold (Ct) values calculated automatically using the Sequence detection software version 1.2.3 (Applied Biosystems). The house keeping genes used were glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (GenBank accession no. NM_002046; forward 5'-ATCCATGACAACCTTGGTATCGTG-3'; reverse 5'-GGCATGGACTGTGGTCATGAG-3'), ribosomal protein large P 0 (RPLP0) (GenBank accession no. NM_001002; forward 5'-GGCGTCCTCGTGGAAGTGACAT-3'; reverse 5'-CAGGGATTGCCACGCAGGGT-3') and 18S (GenBank accession no. NR_003286; forward 5'-CGGAGGTTTCAAGACGATCA-3'; reverse 5'-GGCATCGTTTATGGTCGGAA-3'). These housekeeping genes have been used in previous gene expression analyses in AT and, in agreement with previous studies [19-21], in the current study expression of none of the three housekeeping genes were found to be altered by either obesity or treatment. Individual subject gene expression was normalised to the relevant housekeeping gene [Δ Ct (Δ Ct)]. For comparison of lean vs obese gene expression, the obese group mean fold change relative to the lean control group was calculated using the group mean Δ Ct values in the formula $2^{-\Delta\Delta Ct}$. Differences between lean and obese Δ Ct values were analysed using independent t-tests and a p value less than or equal to 0.05 was considered statistically significant. For pre- vs post-treatment comparisons, individual obese subject post-treatment mRNA expression was calculated as a fold change relative to pre-treatment for each participant using the formula $2^{-\Delta\Delta Ct}$. Differences between pre- and post-treatment Δ Ct values were analysed using paired t-tests. Treatment change (post – pre) was compared with placebo change (post – pre) by independent t-test. A p value less than or equal to 0.05 was considered statistically significant.

Statistical analysis

Statistical analyses were carried out using SPSS software version 18.0. Lean and obese groups were compared at baseline by independent t-test; obese treated groups were analysed by calculating the change (post - pre) and comparing with placebo change (post - pre) also using independent t-tests. Correlations between continuous variables were assessed by Pearson's coefficients for correlations. Logistic regression analysis was used to determine if existing correlations remained once BMI was added to the model.

Individual subject Δ Ct values were used to assess correlations between mRNA expression and continuous variables and, as higher Δ Ct values reflect lower mRNA expression, negative correlations do not represent inverse relationships between variables. Variables not normally distributed were log transformed to natural logarithms prior to analysis to allow for parametric testing. Serum results were obtained from fasting variables and all results are presented as mean \pm standard deviation (S.D). A p value less than or equal to 0.05 was considered statistically significant.

Results

Anthropometric and biochemical characteristics of study participants

Baseline anthropometric and biochemical characteristics of study participants are shown in Table 1. As expected, both BMI and WHR were significantly increased in the obese group relative to the lean group. Additionally, the lean group displayed significantly lower systolic blood pressure (SBP) and diastolic blood pressure (DBP) compared with baseline levels in the obese group. The obese group displayed significantly greater glucose levels at baseline. Nonetheless, levels after 120 minutes of the OGTT were not significantly different between groups, indicating that despite obesity, participants who entered the study had normal glucose tolerance. In support of this, there was no significant difference in HbA_{1c} levels between the groups. There were two surrogate markers of insulin resistance used in the study: fasting insulin levels and HOMA index. Both parameters were significantly lower in the lean participants compared to the obese, signifying insulin resistance within the obese group. Severe dyslipidaemia was an exclusion criterion in the study; this would indicate why TC and LDL-C were similar between the two groups. In keeping with dyslipidaemic features associated with obesity, HDL-C was significantly higher and TGs lower in the lean individuals relative to the obese. Additionally, both the TC/HDL-C ratio and LDL-C/HDL-C ratio were significantly increased with obesity.

Cytokine and peripheral monocyte levels and adipocyte cell surface assessment

As expected, baseline levels of TNF- α were significantly higher in the obese participants compared to the lean (Table 2). Additionally, levels of total and high adiponectin were significantly reduced with obesity (Table 2). There was no significant difference in the high to total adiponectin ratio between the two groups.

The obese group exhibited a significantly higher percentage of peripheral CD14⁺/CD16⁺ monocytes [Lean vs. Obese (%); 13.9 \pm 4.5 vs. 21.1 \pm 5.6, p <0.0001] (Figure 1b) and greater adipocyte cell surface [Lean vs. Obese (μ m²); 1748 \pm 488 vs. 3334 \pm 538, p <0.0001] (Figure 1c) compared to their lean counterparts.

ATM assessment

The percentage of CD68 expressing cells within sc AT [Lean vs. Obese (%); 0.96 \pm 0.69 vs. 3.95 \pm 3.97, p <0.0001] was significantly elevated with obesity (Figure 2a). In parallel, CD68 mRNA expression followed a similar trend, with a 1.62 fold increase in the obese group relative to the lean group (Figure 2b).

Adipose tissue gene expression analysis

The observed obesity-associated increases in circulating TNF- α levels were reflected in a significant increase in sc adipose tissue expression of this cytokine (Figure 3). Expression of SREBP1c, CEBP β , GLUT 4 and IRS-1 were all lower in obese vs lean adipose tissue, however, only the latter was significant (Figure 3). Expression of PPAR γ 2 was similar between lean and obese adipose tissue.

Correlations between adipose markers, peripheral monocytes and CVD risk factors

In agreement with the observation that obesity-associated increases in % of CD68 cells within AT (Figure 2a), BMI and WHR demonstrated positive correlations with % of CD68 expressing cells in sc AT (r =0.595, p =0.001, and r =0.429, p =0.023, respectively).

As can be seen in Figure 4a, adipocyte cell surface correlated significantly with BMI, % of CD68 expressing cells in sc AT and % of peripheral CD14⁺/CD16⁺ monocytes. In addition, adipocyte cell surface correlated significantly with markers of insulin resistance: fasting insulin and HOMA index (Figure 4b). Adipocyte cell surface also correlated significantly with both circulating TNF- α (r =0.455, p =0.008) and adipose tissue TNF- α mRNA levels (Δ Ct r =-0.371, p =0.048), as well as being negatively correlated with total adiponectin (r =-0.411, p =0.017). Only the correlation between adipocyte cell surface and % of CD68 expressing AT cells remained significant (p =0.014) after adjusting for BMI.

The percentage of CD68 expressing cells in sc AT and % of peripheral CD14⁺/CD16⁺ monocytes also demonstrated a significant correlation (Figure 4c); this was lost upon adjusting for BMI. The percentage of CD14⁺/CD16⁺ monocytes also showed a positive correlation with fasting insulin ($r=0.378$, $p=0.048$) and HOMA index ($r=0.422$, $p=0.023$), and a negative correlation with total adiponectin ($r=-0.428$, $p=0.007$), as well as high adiponectin ($r=-0.362$, $p=0.025$). Likewise, the percentage of CD68 expressing AT cells negatively correlated with total (-0.578 , $p=0.001$) and high adiponectin ($r=-0.460$, $p=0.012$). Again, correlations between CD14⁺/CD16⁺ monocytes and indices of insulin resistance and circulating levels of adiponectin isoforms became non-significant when adjusted for BMI, as did correlations between % of CD68 expressing AT cells and adiponectin.

The effects of pioglitazone and fenofibrate on anthropometric and biochemical characteristics

The effects of pioglitazone and fenofibrate on anthropometric and biochemical characteristics are shown in Table 3. Fasting insulin levels and HOMA index both improved significantly following pioglitazone treatment, demonstrating its ability to ameliorate obesity associated insulin resistance. As expected, fenofibrate exhibited its lipid-lowering effects by significantly reducing levels of TC, LDL-C and TG, and improving the TC/HDL-C ratio and the LDL-C/HDL-C ratio. In addition, pioglitazone significantly enhanced HDL-C levels and had similar positive effects to fenofibrate on the TC/HDL-C ratio and the LDL-C/HDL-C ratio.

The effects of pioglitazone and fenofibrate on adiponectin, adipocyte cell surface, ATM and peripheral monocyte numbers

With respect to adiponectin, pioglitazone augmented levels of total [Pre vs. Post ($\mu\text{g/mL}$); 5.5 ± 1.4 vs. 10.4 ± 5.7 , $p<0.0001$] and high adiponectin [Pre vs. Post ($\mu\text{g/mL}$); 2.8 ± 1.1 vs. 6.9 ± 4.6 , $p<0.0001$], and additionally, pioglitazone treatment improved the high to total adiponectin ratio (Pre vs. Post; 0.49 ± 0.09 vs. 0.62 ± 0.11 , $p<0.0001$).

Neither pioglitazone nor fenofibrate treatment resulted in any significant changes in CD68 mRNA expression or the percentage of CD68 expressing cells within AT, nor did either treatment have any effect on the percentage of peripheral CD14⁺/CD16⁺ monocytes. Following pioglitazone treatment there was, however, a significant increase in adipocyte cell surface [Pre vs. Post (μm^2); 3330 ± 712 vs. 3655 ± 712 , $p=0.05$].

The effects of pioglitazone and fenofibrate on adipose tissue gene expression

Fenofibrate was without effect on the expression levels of any of the genes examined. Pioglitazone significantly increased expression of CEBP β (Figure 5; 4.26 fold). Pioglitazone treatment also resulted in increased expression of SREBP1c, PPAR γ 2, GLUT 4, and IRS-1, albeit that none of these increases reached significance (Figure 5).

Following pioglitazone treatment, CEBP β expression exhibited significant positive correlations with IRS-1 ($r=0.979$, $p<0.0001$) and SREBP1c ($r=0.976$, $p<0.0001$) mRNA levels, and a significant negative correlation with CD68 ($r=-0.822$, $p=0.045$) mRNA expression.

Discussion

Using a cohort of lean and obese glucose tolerant subjects this study is the first to simultaneously investigate obesity-associated changes in both peripheral blood CD14⁺/CD16⁺ monocytes and ATM. The results obtained indicate that obesity is associated with increased peripheral CD14⁺/CD16⁺ monocyte and sc ATM number, as well as increased sc adipocyte cell surface, reduced circulating adiponectin levels and lower sc AT expression of genes involved in macrophage phenotype, adipogenesis and glucose metabolism. Moreover, pioglitazone treatment was found to result in significant improvements in insulin resistance and lipid profile, changes which occurred in parallel with significant increases in sc AT expression of CEBP β mRNA, adipocyte cell surface and adiponectin levels. Taken together these results indicate that obesity-associated changes in CD14⁺/CD16⁺ monocyte levels and adipose function (as reflected in reduced systemic levels of adiponectin) are evident prior to the presence of impaired glucose tolerance, and that therapeutic interventions with the ability to target the latter may prove beneficial in reducing CVD risk and the development of T2D.

Prediabetic individuals are defined as those with impaired glucose tolerance (2hr glucose 140-199 mg/dL [7.8-11.0 mmol/L]) or impaired fasting glucose (fasting glucose concentration 110-125 mg/dL [5.6-6.9 mmol/L]) or an HbA_{1c} of 5.7-6.4% [22]. A strength of the current study is that the obese subjects recruited did not have impaired glucose tolerance (2hr glucose 6.4 \pm 1.0 mmol/L; fasting glucose concentration 5.3 \pm 0.4 mmol/L; HbA_{1c} 5.3 \pm 0.3%), thereby, enabling obesity-associated changes in primary and secondary endpoints to be investigated and the impact of PPAR agonist treatment on these endpoints to be evaluated. Cardioprotective strategies, including lifestyle modification, have been shown to be most effective in averting or delaying the onset of diabetes when administered at this early stage in the hyperglycaemia/diabetes continuum [23]. The current study has provided evidence that preventative strategies are also effective when administered prior to the development of impaired glucose tolerance.

Subcutaneous and visceral ATM number increase in obesity and evidence is accumulating that ATM are responsible for potentiating the chronic inflammatory processes of obesity [1, 2]. The origin of ATM remains to be fully elucidated, it has been reported that these arise from adipokine-dependent extravasated peripheral blood monocytes and evidence has also been presented that multi-potent adipocyte stem cells and pre-adipocytes can differentiate into macrophages [24, 25]. Both sc and visceral ATM number have been reported to correlate with clinical parameters of obesity and its comorbidities [18]. The results of the current study concur with previous reports, the percentage of CD68 expressing cells within AT was significantly elevated in obesity, while CD68 mRNA expression followed a similar trend. Moreover, BMI and WHR demonstrated positive correlations with the percentage of CD68 expressing cells in sc AT. The percentage of CD68 expressing cells also negatively correlated with total and high adiponectin levels, indicating that ATM infiltration is associated with reduced adipocyte function. Adipocyte enlargement is a strong, direct predictor of ATM recruitment and accumulation [26]. Macrophages have been detected in the sc and visceral AT of obese patients, in which they surround the dead adipocyte in a crown-like arrangement [27]. Given the evidence that adipocyte hypertrophy is a potential stimulus for ATM infiltration, it is of interest that, in the current study, adipocyte cell surface correlated significantly with both % of CD68 expressing cells in sc AT and % of peripheral CD14⁺/CD16⁺ monocytes. Given the observed obesity-associated increases in the CD14⁺/CD16⁺ peripheral monocyte population, the significant correlation between % of CD68 expressing cells in sc AT and % of peripheral CD14⁺/CD16⁺ monocytes, and the evidence supporting a role for this population in vascular inflammation, it is tempting to speculate that these represent a source of the increased ATM number in sc AT, however, clearly this is an area that requires further analysis.

The observation in the current study that pioglitazone and fenofibrate normalised obesity-associated insulin resistance and/or dyslipidaemia is as would be expected, indeed, in an earlier study similar results were reported by our group [11]. The observation that pioglitazone achieved this without significantly changing % of peripheral CD14⁺/CD16⁺ monocytes or ATM number, but by significantly increasing adipocyte cell surface and improving adipocyte function is a novel one.

Taken together, these results suggest a model whereby modulation of adipocyte function, in the absence of effects on CD14⁺/CD16⁺ monocyte expansion or ATM number, results in cardioprotective outcomes. While Di Gregorio and co workers [28] have reported a decrease in ATM following pioglitazone treatment, their cohort received a higher dose than used in the current trial (45 mg/day), moreover, their subjects had impaired glucose tolerance and were mixed gender. Hammarstedt and co-workers have reported that 3 week treatment with a similar dose of pioglitazone in overweight, insulin resistant, glucose tolerant subjects had no effect on adipocyte cell surface, or indeed, in agreement with the results of the current study, adipose macrophage infiltration markers [29]. In contrast to the current study, however, short term pioglitazone treatment was found to be without effect on lipid levels [29]. PPAR γ agonists are reported to promote free fatty acid uptake and storage in adipocytes, thereby preventing lipotoxic trauma to liver and muscle and ameliorating insulin resistance. In support of this, pioglitazone has been demonstrated to increase sc adipose mass [30]. Moreover, it has been reported that congenital adrenal hyperplasia-associated insulin resistance was improved by pioglitazone treatment, and that this improvement in insulin sensitivity was associated with enlargement of sc adipocytes [12].

A further novel finding of the current study is that, following pioglitazone treatment, sc AT CEBP β expression was significantly increased. Expression of CEBP β , SREBP1c, GLUT 4 and IRS-1 were all lower in obese vs lean sc AT, the latter being significantly reduced. These results are in keeping with an obesity-associated impairment in adipogenesis [1]. CEBP β has been recently identified as a playing a central role in PPAR γ -mediated gene regulation in both adipocytes and macrophages, two cell types which predominate in obese sc AT [31, 32]. In addition, evidence has been presented that CEBP β plays a non-PPAR γ -dependent role in mitotic clonal expansion during adipogenesis [33]. The observation that pioglitazone upregulates CEBP β is interesting given the central role of this transcription factor in adipogenesis and macrophage polarisation, and provides evidence that pioglitazone, in addition to increasing the function/size of mature adipocytes, promotes adipogenesis and may facilitate macrophage polarisation to the alternative, anti-inflammatory phenotype. It will be of interest in future studies to determine if pioglitazone-dependent CEBP β upregulation is reflected in increased protein levels of this transcription factor and whether this is associated with an increase in anti-inflammatory, and a decrease in proinflammatory, cytokine expression, as demonstrated in our previous study [11]. Pioglitazone treatment also resulted in increased, albeit non-significant, sc adipose expression of SREBP1c, PPAR γ 2, GLUT 4, and IRS-1. These results are in broad agreement with the findings of Hammarstedt et al [29], and further support a model whereby pioglitazone treatment facilitates adipocyte terminal differentiation.

In conclusion, the current study has found evidence that increased adipocyte cell surface, expansion of the CD14⁺/CD16⁺ monocyte population and ATM number, and compromised AT function occurs in obese individuals prior to the development of impaired glucose tolerance. Further, significant associations between adipocyte cell surface and increased peripheral monocyte and ATM numbers support the role of adipocyte enlargement as an initiating stimulus in adipose inflammation/dysfunction. The finding that improvements in obesity-associated insulin resistance following pioglitazone were associated with increased adipocyte cell surface and systemic adiponectin levels, supports the centrality of AT to the cardiometabolic derangement underlying the development of T2D and CVD.

Author contribution

LAP, AMG, DRM, ISY and ERT and were involved in the study conception, design and management, data interpretation and drafting of the manuscript. PC, CK and JMP were involved in the recruitment of participants, sample collection and analysis, data analysis and manuscript preparation.

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Table 1 Anthropometric and biochemical characteristics of study participants at baseline

Data are expressed as means \pm SD. Lean vs. obese groups were compared at baseline using independent t-tests.

Characteristics	Lean controls (n=16)	Obese (n=37)	Differences in Means (95% CI)	<i>p</i> value
Age (years)	50 \pm 7	47 \pm 7	4 (−0.09, 7.83)	ns
BMI (kg/m ²)	23.9 \pm 1.4	35.0 \pm 4.0	−11.1 (−12.57, −9.57)	<0.0001
WHR	0.88 \pm 0.10	1.01 \pm 0.05	−0.12 (−0.17, −0.08)	<0.0001
SBP (mmHg)	117 \pm 9	129 \pm 12	−12 (−18.09, −4.98)	0.001
DBP (mmHg)	74 \pm 7	82 \pm 8	−8 (−12.53, −3.62)	0.001
Fasting glucose (mmol/L)	5.1 \pm 0.4	5.3 \pm 0.4	−0.2 (−0.45, −0.002)	0.047
2hr OGTT (mmol/L)	5.8 \pm 1.3	6.4 \pm 1.0	−0.6 (−1.25, 0.07)	ns
Fasting insulin (mU/l)	6.6 \pm 3.0	12.9 \pm 4.9	−6.3 (−8.90, −3.77)	<0.0001
HOMA index	1.4 \pm 0.6	3.2 \pm 1.4	−1.7 (−2.36, −1.10)	<0.0001
HbA _{1c} (%)	5.2 \pm 0.2	5.3 \pm 0.3	−0.1 (−0.31, 0.09)	ns
TC (mmol/L)	5.0 \pm 0.8	5.3 \pm 0.6	−0.4 (−0.78, 0.07)	ns
LDL-C (mmol/L)	3.1 \pm 0.9	3.4 \pm 0.5	−0.3 (−0.79, 0.19)	ns
HDL-C (mmol/L)	1.46 \pm 0.27	1.22 \pm 0.24	0.24 (0.09, 0.39)	0.002
TG (mmol/L)	1.00 \pm 0.40	1.60 \pm 0.64	−0.60 (−0.95, −0.25)	0.001
TC/HDL-C ratio	3.55 \pm 1.14	4.45 \pm 0.73	−0.89 (−1.54, −0.25)	0.009
LDL-C/HDL-C ratio	2.24 \pm 0.98	2.82 \pm 0.55	−0.58 (−1.13, −0.04)	0.038

Table 2 Serum levels of inflammatory markers of study participants at baseline

Data are expressed as means \pm SD. Lean vs. obese groups were compared at baseline using independent t-tests.

Inflammatory markers	Lean controls (n=15)	Obese (n=37)	Differences in Means (95% CI)	<i>p</i> value
TNF- α (pg/mL)	1.62 \pm 0.36	2.14 \pm 0.91	-0.53 (-1.01, -0.04)	0.017
Total Adiponectin (μ g/mL)	10.5 \pm 4.5	6.3 \pm 2.1	4.2 (1.59, 6.74)	<0.0001
High Adiponectin (μ g/mL)	4.6 \pm 2.6	3.1 \pm 1.4	1.5 (0.01, 3.04)	0.022
High/Total Adiponectin ratio	0.42 \pm 0.10	0.47 \pm 0.11	-0.05 (-0.11, 0.02)	ns

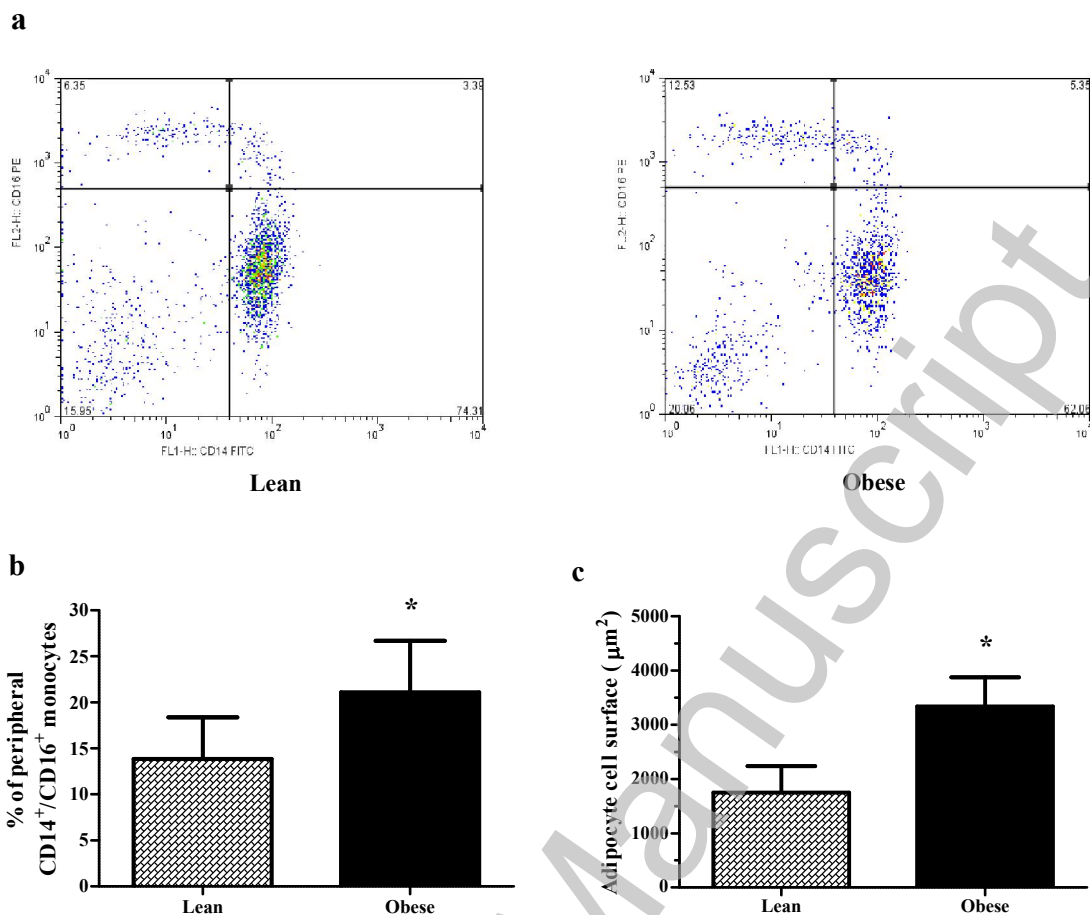


Figure 1 Baseline analysis of CD14+CD16+ peripheral monocyte populations and adipocyte cell surface in lean and obese groups at baseline. Representative scatter dot plots showing CD14+CD16+ peripheral monocyte populations in lean and obese groups at baseline. Peripheral blood mononuclear cells were isolated by Histopaque and labelled with Fluorescein Isothiocyanate (FITC)-conjugated anti-CD14 and Phycoerythrin (PE)-conjugated anti-CD16. Fixed monocytes were identified by forward and side scatter properties. Fluorescence data were collected on 10,000 cells and analysed using CellQuest Pro software. The percentage of CD14+CD16+ cells was calculated by combining the top left and right quadrants of the gated monocyte population (a). Mean percentage of peripheral CD14⁺/CD16⁺ monocytes (b), and mean adipocyte cell surface (μm²) (c) in lean (n=9-15) and obese (n=23-25) groups at baseline. Error bars indicate S.D. Lean vs. obese groups were compared at baseline using independent t-tests: **p*<0.05.

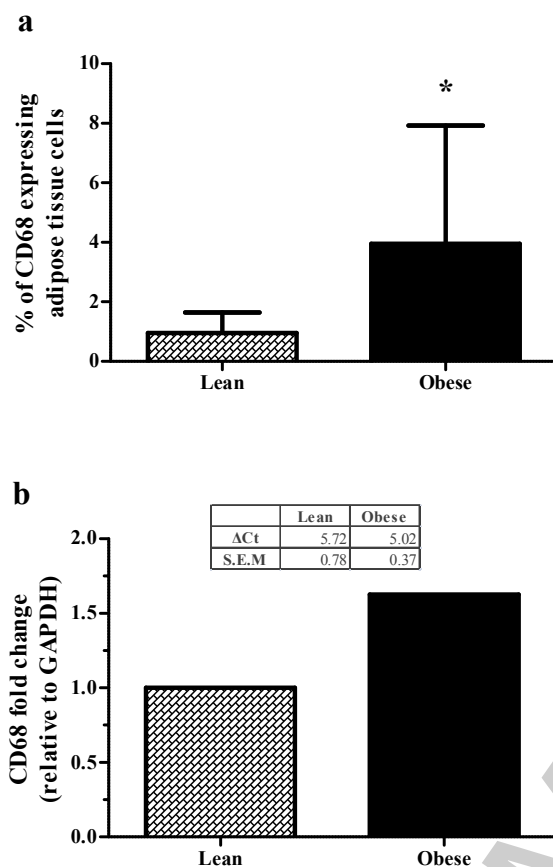
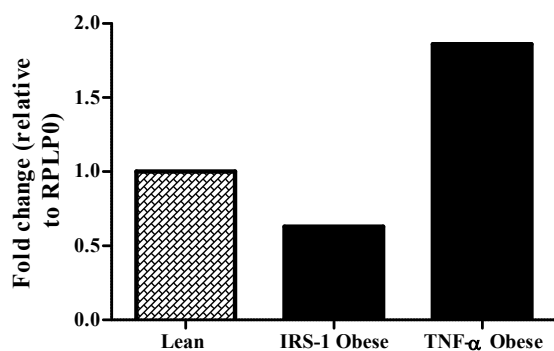


Figure 2 Comparison of mean percentage (\pm S.D) of CD68 expressing AT cells (a), and mean CD68 mRNA expression (b) in lean (n=7-13) versus obese (n=24-28) groups

Individual subject CD68 mRNA expression was normalised to the housekeeping gene GAPDH using the formula ΔC_t and the mean fold change relative to the lean control group calculated using the formula $2^{-\Delta\Delta C_t}$ (b). Data shown as group mean fold change and statistical analyses were performed on the individual ΔC_t values. Differences between lean and obese groups at baseline were analysed using independent t-tests: * $p < 0.05$.

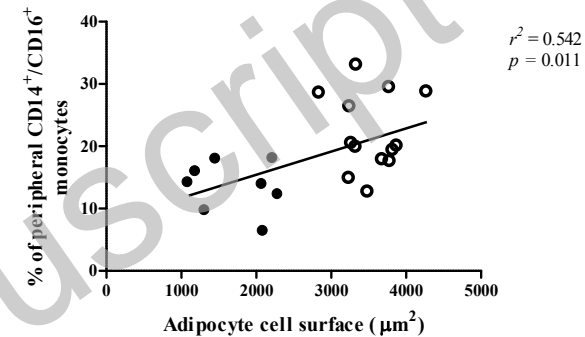
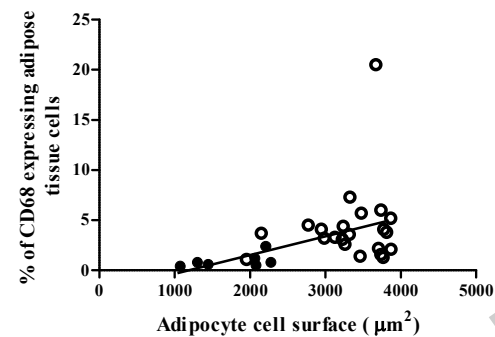
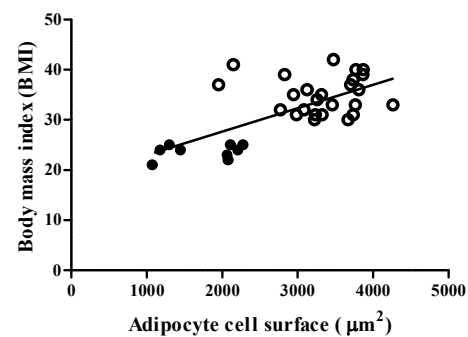


Gene		Lean	Obese
IRS-1	ΔCt	3.25	3.92*
	S.E.M	0.24	0.14
TNF- α	ΔCt	11.67	10.78*
	S.E.M	0.44	0.18
SREBP1c	ΔCt	4.88	5.42
	S.E.M	0.41	0.22
CEBP β	ΔCt	5.82	6.29
	S.E.M	0.29	0.18
PPAR γ 2	ΔCt	1.51	1.62
	S.E.M	0.17	0.09
GLUT 4	ΔCt	7.48	8.01
	S.E.M	0.33	0.15

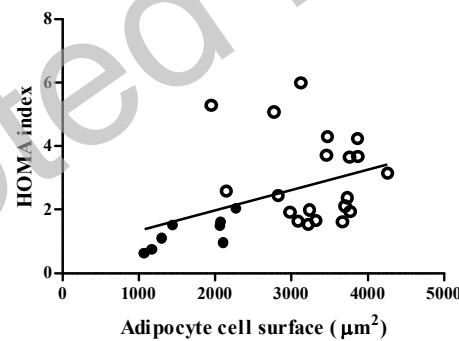
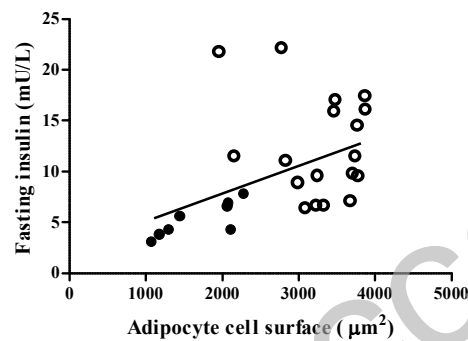
Figure 3 Comparison of adipose gene expression in lean (n=12) versus obese (n=29) groups

Individual subject IRS-1 and TNF- α mRNA expression was normalised to the housekeeping gene RPLP0 using the formula ΔCt and the mean fold change relative to the lean control group calculated using the formula $2^{-\Delta\Delta Ct}$. Data shown as group mean fold change and statistical analyses were performed on the individual ΔCt values. Differences between lean and obese groups at baseline were analysed using independent t-tests: * $p < 0.05$.

a



b



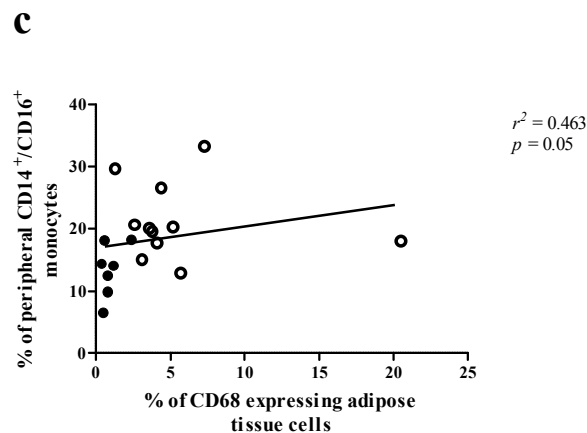


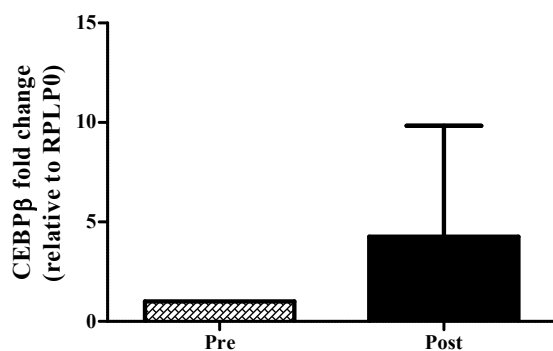
Figure 4 Correlations between adipocyte cell surface (μm^2) and BMI, % of CD68 expressing cells and % of peripheral CD14⁺/CD16⁺ monocytes (a); correlations between adipocyte cell surface (μm^2) and fasting insulin (mU/L) and HOMA index (b); correlation between % of CD68 expressing cells and % of peripheral CD14⁺/CD16⁺ monocytes (c); ○ Lean ● Obese

Correlations between continuous variables were assessed by Pearson's coefficients for correlations.

Table 3 Anthropometric and biochemical characteristics of obese participants pre and post treatment

Data are expressed as a mean \pm SD. Obese treated groups were analysed by calculating change (post – pre) and comparing with placebo change (post – pre) using independent t-test: * p <0.05; † p <0.01.

Characteristics	Pioglitazone (n = 6-8)			Fenofibrate (n = 7-11)			Placebo (n = 5-9)		
	Pre	Post	Mean % change	Pre	Post	Mean % change	Pre	Post	Mean % change
BMI (kg/m ²)	36.6 \pm 3.8	36.5 \pm 3.8	0 %	34.6 \pm 4.7	35.0 \pm 5.7	1 %	34.1 \pm 3.7	34.7 \pm 3.6	2 %
WHR	1.02 \pm 0.05	1.01 \pm 0.05	-1 %	1.02 \pm 0.08	1.00 \pm 0.06	-2 %	0.97 \pm 0.03	0.97 \pm 0.05	0 %
SBP (mmHg)	137 \pm 10	136 \pm 15	-1 %	126 \pm 8	119 \pm 9	-5 %	130 \pm 14	125 \pm 15	-4 %
DBP (mmHg)	85 \pm 8	88 \pm 8	4 %	79 \pm 10	80 \pm 6	3 %	82 \pm 4	81 \pm 6	-2 %
Fasting glucose (mmol/L)	5.1 \pm 0.4	5.1 \pm 0.2	0 %	5.4 \pm 0.3	5.1 \pm 0.4	-5 %	5.3 \pm 0.5	5.3 \pm 0.4	1 %
2hr glucose (mmol/L)	6.2 \pm 0.7	5.9 \pm 1.3	-3 %	6.4 \pm 1.0	6.3 \pm 1.1	-1 %	6.8 \pm 1.3	6.7 \pm 1.4	2 %
Fasting insulin (mU/l)	15.6 \pm 5.1	12.0 \pm 2.9*	-16 %	13.0 \pm 5.1	14.4 \pm 8.2	9 %	9.9 \pm 3.4	12.5 \pm 5.7	25 %
HOMA index	3.6 \pm 1.4	2.7 \pm 0.7*	-15 %	3.2 \pm 1.6	3.2 \pm 1.7	2 %	2.4 \pm 1.0	3.0 \pm 1.4	27 %
HbA1c (%)	5.2 \pm 0.2	5.2 \pm 0.2	0 %	5.4 \pm 0.3	5.3 \pm 0.2	-1 %	5.3 \pm 0.3	5.3 \pm 0.3	1 %
TC (mmol/L)	4.9 \pm 0.4	4.9 \pm 0.8	-1 %	5.5 \pm 0.7	4.8 \pm 0.6*	-13 %	5.3 \pm 0.8	5.3 \pm 0.5	1 %
LDL-C (mmol/L)	3.1 \pm 0.4	3.0 \pm 0.9	-5 %	3.7 \pm 0.6	3.1 \pm 0.6*	-14 %	3.1 \pm 0.5	3.3 \pm 0.4	9 %
HDL-C (mmol/L)	1.15 \pm 0.22	1.19 \pm 0.20*	5 %	1.28 \pm 0.23	1.22 \pm 0.23	-4 %	1.26 \pm 0.35	1.14 \pm 0.30	-10 %
TG (mmol/L)	1.48 \pm 0.39	1.51 \pm 0.85	7 %	1.31 \pm 0.42	1.02 \pm 0.39†	-21 %	2.01 \pm 0.83	2.35 \pm 1.37	13 %
TC/HDL-C ratio	4.43 \pm 0.91	4.20 \pm 1.00*	-5 %	4.40 \pm 0.69	4.04 \pm 0.97†	-8 %	4.38 \pm 0.77	4.90 \pm 0.99	12 %
LDL-C/HDL-C ratio	2.81 \pm 0.72	2.59 \pm 1.00*	-9 %	2.91 \pm 0.49	2.64 \pm 0.79*	-9 %	2.50 \pm 0.42	2.94 \pm 0.67	18 %



Gene		Pre	Post
CEBPβ	ΔCt	6.43	5.31*
	S.E.M	0.37	0.40
SREBP1c	ΔCt	5.79	5.14
	S.E.M	0.42	0.46
PPARγ2	ΔCt	1.74	0.86
	S.E.M	0.21	0.46
GLUT4	ΔCt	8.29	7.77
	S.E.M	0.32	0.47
IRS-1	ΔCt	3.78	3.24
	S.E.M	0.21	0.31

Figure 5 Comparison of adipose gene expression in obese (n=7) subjects pre- and post-pioglitazone treatment

Post-treatment mRNA expression was calculated as a fold change relative to pre-treatment using the formula $2^{-\Delta\Delta Ct}$. Data shown as mean fold change (\pm S.D) and statistical analyses were performed on the individual ΔCt values. Pioglitazone change (post – pre) was compared with placebo change (post – pre) using independent t-tests: *p<0.05.